Aneuploid sperm formation in rainbow trout exposed to the environmental estrogen 17α-ethynylestradiol

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Environmental contaminants that mimic native estrogens (i.e., environmental estrogens) are known to significantly impact a wide range of vertebrate species and have been implicated as a source for increasing human male reproductive deficiencies and diseases. Despite the widespread occurrence of environmental estrogens and recognized detrimental effects on male vertebrate reproduction, no specific mechanism has been determined indicating how reduced fertility and/or fecundity is achieved. Previous studies show that male rainbow trout, *Oncorhynchus mykiss*, exposed to the environmental estrogen 17α-ethynylestradiol (EE2) before gamete formation and fertilization produce progeny with significantly reduced embryonic survival. To determine whether this observed decrease results from sperm chromosome alterations during spermatogenesis, male rainbow trout were exposed to 10 ng of EE2/l for 50 days. After exposure, semen was collected and sperm aneuploidy levels analyzed with two chromosome markers by fluorescent *in situ* hybridization. *In vitro* fertilizations were also conducted by using control and exposed sperm crossed to eggs from an unexposed female for offspring analysis. Evaluations for nucleolar organizer region number and karyotype were performed on developing embryos to determine whether sperm aneuploidy translated into embryonic aneuploidy. Results conclusively show increased aneuploid sperm formation due to EE2 exposure. Additionally, embryonic cells from propagated progeny of individuals possessing elevated sperm aneuploidy display high levels of embryonic aneuploidy. This study concludes that EE2 exposure in sexually developing male rainbow trout increases levels of aneuploid sperm, providing a mechanism for decreased embryonic survival and ultimately diminished reproductive success in EE2 exposed males.

aneuploidy | EE2 | xenoestrogens

Endocrine disrupting chemicals are found ubiquitously worldwide due to human environmental contamination (1–4). These chemicals comprise several different classes and those with an estrogenic mode of action (i.e., environmental estrogens) are of major concern. In industrialized countries concerns about human health impacts resulting from environmental estrogens have been mounting. These concerns largely resulted from a meta-analysis of sperm counts indicating a nearly 50% reduction between 1940 and 1990 (5). This finding proved controversial with additional analyses supporting (6–11) and contradicting (12–14) the original finding. Additional reports suggest that a rapid increase in testicular germ cell cancers, cryptorchidism, and other congenital anomalies in developed countries may be related to increased environmental estrogen exposure (15–18). Additionally, recent animal model studies have indicated that environmental estrogens are capable of altering epigenetic patterns during early development with resulting gene expression changes, increased cancer susceptibility, and congenital aberrations as well as diminished reproductive capacity, compounding human health concerns (19, 20). These studies, and others, have led to the hypothesis that reduced fertility and/or increased reproductive anomalies result from exposure to environmental estrogens.

Environmental estrogens have a wide variety of chemical structures but are grouped together based on their ability to mimic natural estrogen by interfering with/or binding directly to estrogen receptors (21–24). Through these actions environmental estrogens affect vertebrate reproduction across a wide range of doses causing reduced fertility and fecundity, altered reproductive behavior, gonad morphological changes, and decreased embryonic survival (25–34). Although both sexes are affected, males exhibit the highest degree and number of detrimental effects. Specific male defects caused by environmental estrogen exposures in vertebrates include: intersex (25, 29), diminished sperm count (27, 35–37), genital tract alterations (38), increased germ cell apoptosis (30, 33), and male induced embryonic mortality (31, 39).

Despite the evidence for significant effects of environmental estrogen exposure on male vertebrates, no specific mechanism of action has been determined to explain how reduced fertility and/or fecundity of morphologically and physiologically normal individuals are achieved. Although most rodent model studies show decreased sperm production and/or increased apoptosis of germ cells after exposure (27, 30, 33, 35), others have failed to confirm these findings (40). Additionally, recent *in vitro* exposures of human spermatozoa to catechol estrogens (e.g., quercetin, diethylstilbestrol and pyrocatechol) indicate an impact on sperm DNA integrity through altered redox cycling, but estrogen (17β-estradiol) and other estrogen analogues (nonylphenol and BPA) do not show this effect (41). Despite this *in vitro* finding using spermatozoa, how these compounds would affect spermatogenesis *in vivo* is unknown.

Fish studies in which male rainbow trout (*Oncorhynchus mykiss*) are exposed *in vivo* to the environmental estrogen 17α-ethynylestradiol (EE2) show no defects in either testis morphology or sperm motility, but exhibit significantly reduced progeny survival when exposed as late stage juveniles during final sexual maturation (31, 42). In these studies, which specifically evaluated the effect of EE2 exposure on the male germ cell at environmentally relevant concentrations ($\approx$10 ng/l) during spermatogenesis (i.e., meiosis), the problem was attributed to qualitative sperm defects. Further evaluations led to the consideration of a possible genetic link affecting embryonic survival. This new hypothesis is based on previous studies in which rainbow trout sperm with fragmented DNA (UV irradiated) were used to fertilize eggs *in vitro*, resulting in an observed increase in embryonic aneuploidy (43, 44). Results from these experiments show a significant reduction in the survival of

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embryos produced with UV irradiated sperm compared with control embryos propagated by using un-irradiated sperm (45). This study indicates that sperm chromosome damage leads to a pattern of embryo death similar to that observed when male parents are exposed to EE2 (31, 42). Additionally, evidence exists that exposure of female mice to low levels of BPA and of maturing oocytes to supraphysiological levels of 2-methoxyestradiol, causes increases in aneuploid oocyte formation (46, 47), although the BPA studies could not be reproduced by another study (40). These observations led us to propose that exposure of sexually maturing male rainbow trout to EE2 results in aneuploid sperm formation, which causes a significant proportion of embryos to die shortly after fertilization. This study used the previously established male rainbow trout experimental model, with fish exposed during the actively meiotic, midspermatogenic time point with spermatocytes and spermatids predominant in the gonad before final sexual maturation, to determine: (*i*) whether an environmentally relevant concentration of EE2 during late spermatogenesis results in the formation of aneuploid sperm and (*ii*) whether progeny produced from males exposed to EE2 during late spermatogenesis exhibit increased levels of aneuploidy.

Results

Five clonal YY male rainbow trout each were exposed as late stage juveniles immediately before sexual maturation (6,700°d; ≈1 year 9 months of age) to a nominal concentration of 10 ng of EE2/l for 50 days or to methanol only (solvent control), respectively. The mean measured daily in-flow water concentration was 16.1 ng of EE2/l with a mean GCMS measured EE2 level of 8.9 ng of EE2/l, both falling within 50–100% of nominal values. After exposure semen was collected from all sexually mature individuals and cryopreserved for later analyses (four control and four exposed). The remaining two individuals, one per treatment, did not produce sperm or exhibit secondary sexual characteristics and possessed immature testes upon postmortem examination. This is not unusual as the majority of male rainbow trout typically attain sexual maturity for the first time at two years of age but a small proportion do not until three years of age.

Fluorescent *in situ* hybridization (FISH) analysis on cryopreserved sperm was performed using two probes, an 18s rDNA probe (Vysis green) hybridizing to chromosome 20 and a 5s rDNA probe (Vysis orange) hybridizing to the Y chromosome (48). Fig. 1 shows examples of normal and aneuploid sperm nuclei evaluated by using FISH for this study. Quantitatively FISH analysis with both probes revealed significant increases in levels of sperm aneuploidy for exposed but not control individuals (Fig. 2). Average combined sperm aneuploidy levels for both probes were 1.2% (control) and 29.1% (exposed), and were significantly different ($P < 0.0001$). Although both Y chromosome and chromosome 20, based on FISH analysis, were equally represented in controls, chromosome 20 aneuploidy was more frequently observed in exposed individuals compared with Y chromosome aneuploidy (Table 1).

In vitro fertilizations were performed by using cryopreserved semen and freshly collected eggs from a single, unexposed female to determine offspring aneuploidy levels. Embryo analysis consisted of nucleolar organizer region (NOR) silver staining and karyotype counts. NOR analysis was performed on 25 control and 30 exposed individuals (Fig. 3). Rainbow trout have a single NOR on chromosome 20 (48, 49). Analysis revealed cell nuclei from the control embryos predominantly exhibiting two NORs per nucleus, consistent with normal diploid rainbow trout. Contrary to this finding, only 73% of the embryos propagated from males exposed to EE2 exhibited two NORs. Of the remaining 27%, 17% expressed one NOR per nucleus, and 10% possessed predominately three NORs per cell nucleus, consistent with being haploid (or hypoploid) and triploid (or hyperploid), respectively (Fig. 4*A*).

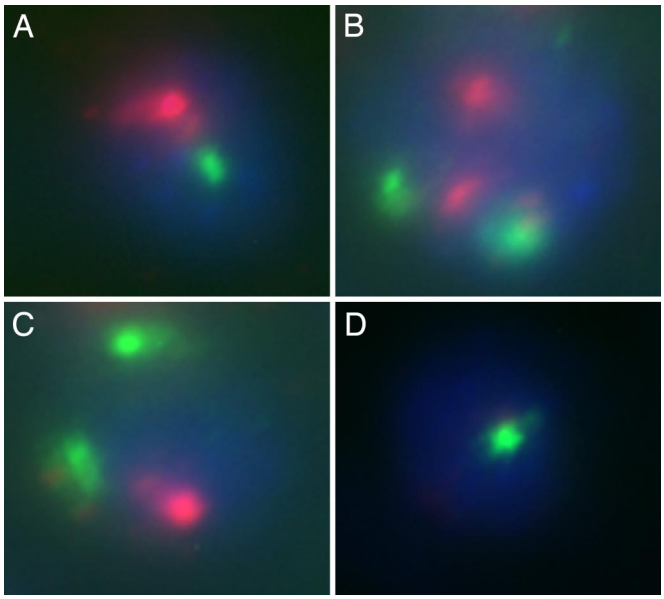

Fig. 1. Representative photomicrographs of normal and aneuploid rainbow trout sperm nuclei with chromosomes identified by using fluorescent *in situ* hybridization. (*A*) Normal, haploid nucleus (stained blue) from a control sperm with one chromosome 20 (green probe) and one sex chromosome (red probe). (*B* and *C*) Examples of hyperploid sperm nuclei and (*D*) a hypoploid sperm nucleus from EE2 exposed fish.

Karyotype analysis on 10 randomly selected embryos from the control group revealed a chromosome count of 62 with 104 chromosome arms, which is consistent with previous reports for this population (50). An embryo chromosome count was considered aneuploid if it deviated from controls for either a whole chromosome or a chromosome arm number with a minimum 10 of 15 individual spreads possessing the same count required to determine ploidy (Fig. 5). Analysis of 24 embryos from EE2 exposed males revealed only 42% with normal karyotypes. Among individuals deemed aneuploid 42% were hypoploid and 16% hyperploid. Exposed group embryonic chromosome counts ranged from 57 to 90 with associated arm numbers ranging from 97 to 153, respectively (Fig. 4*B*). Ten exposed group embryos were analyzed for both NOR and karyotype. Among these ten

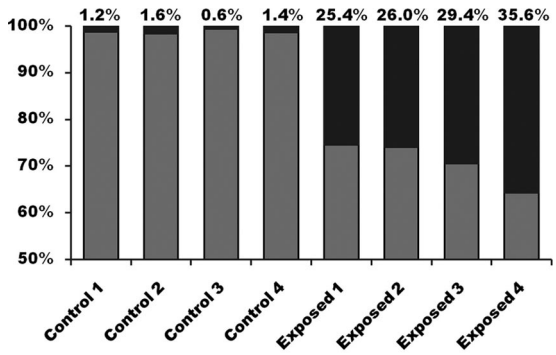

Fig. 2. Combined total percent of normal (gray) and aneuploid (black) sperm collected from control and exposed males evaluated by fluorescent *in situ* hybridization. The percentage of aneuploid sperm for each individual exhibited is given above each bar. Differences between groups were statistically significant at $P < 0.0001$.

Table 1. Summary of sperm chromosome hyperploidy/hypoploidy frequency distributions in male rainbow trout exposed to the solvent vehicle (Control) or 17α-ethynylestradiol (Exposed), determined by fluorescent *in situ* hybridization

	Frequency of hyperploidy, *f*% (n)				Frequency of hypoploidy, *f*% (n)		
	Y	20	Both	Other	Y	20	Both
Control 1	0	0.006 (3)	0.002 (1)	0	0.002 (1)	0.002 (1)	0
Control 2	0.002 (1)	0.004 (2)	0	0	0.002 (1)	0.006 (3)	0.002 (1)
Control 3	0	0.002 (1)	0	0	0	0.002 (1)	0.002 (1)
Control 4	0	0.006 (3)	0	0	0.004 (2)	0.004 (2)	0
Exposed 1	0.030 (15)	0.088 (44)	0.006 (3)	0.002 (1)	0.038 (19)	0.078 (39)	0.012 (6)
Exposed 2	0.042 (21)	0.086 (43)	0.008 (4)	0	0.048 (25)	0.062 (31)	0.014 (7)
Exposed 3	0.054 (27)	0.104 (52)	0.004 (2)	0	0.042 (21)	0.080 (40)	0.010 (5)
Exposed 4	0.046 (23)	0.136 (68)	0.010 (5)	0.004 (2)	0.030 (15)	0.114 (57)	0.016 (8)

Y, Y chromosome; 20, chromosome 20; Both, Y chromosome and chromosome 20; Other, more than two of either probe per nuclei. A total of 500 sperm per individual were examined to determine distributions of hypoploid and hyperploid sperm.

individuals five were confirmed to be hypoploid or hyperploid by combined NOR staining and karyotype analysis.

Discussion

Late stage juvenile exposures of sexually maturing male rainbow trout to an environmental estrogen, EE2, have previously been shown to affect their progeny survival with significant mortality during early embryonic development (31, 42). To determine whether these previous findings result from a genetic defect carried forward in the sperm, studies assessing sperm aneuploidy were undertaken. The present study conclusively provides, for the first time in a male vertebrate, evidence of increased aneuploid sperm formation resulting from EE2 exposure. Further, elevated levels of embryonic aneuploidy occurred in progeny from fathers exposed to EE2 with high levels of sperm aneuploidy, providing a possible mechanism for previously observed reductions in progeny survival.

Aneuploidy is a condition where a reduced or additional number of chromosomes, or portions of chromosomes, occurs within a cell as a result of improper chromosome segregation during either mitosis or meiosis. In fertile, nonpathological human semen the percentage of aneuploid sperm averages between 0.5% to 1.2% (51, 52). This corresponds well with the average of 1.2% aneuploid sperm found in male rainbow trout semen samples from the control group in this study and may indicate a general low level of aneuploid sperm formation in vertebrates. Contrasting these stable sperm chromosome levels, our results indicate significantly elevated levels of chromosomally abnormal sperm in EE2 exposed male rainbow trout, a condition similar to that observed in humans and rodent models exposed to chemotherapeutic agents and radiation (53, 54). Several mechanisms and/or predisposing factors of aneuploid sperm formation are known for humans. These include the presence of translocations in the genome (a rare event), which can affect not only the chromosome to which the translocation occurs but additional chromosomes through an interchromosomal effect (55, 56) and decreased or altered meiotic recombination (57–59). One specific point where meiotic recombination can be disturbed is during M-phase, when spindles are forming and chromosome segregation occurs. This point is of specific interest as only meiotic divisions were exposed to EE2 in our study. Although such disturbances can affect all chromosomes, it has been observed that chromosomes with fewer

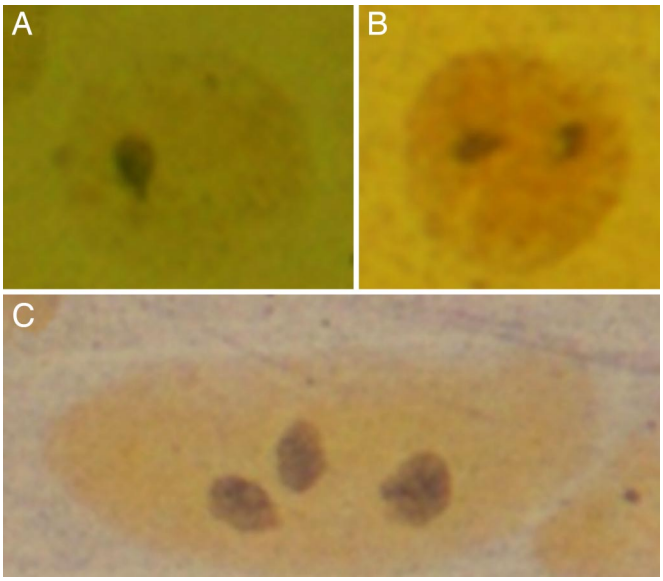

Fig. 3. Representative photomicrographs of embryonic cell nuclei silver stained to reveal nucleolar organizer regions (NOR). (*A*) Monosomic cell with only one NOR stained chromosome 20. (*B*) Representative normal disomic cell with two stained NOR chromosome 20 s. (*C*) Trisomic condition with three NOR stained chromosome 20s present.

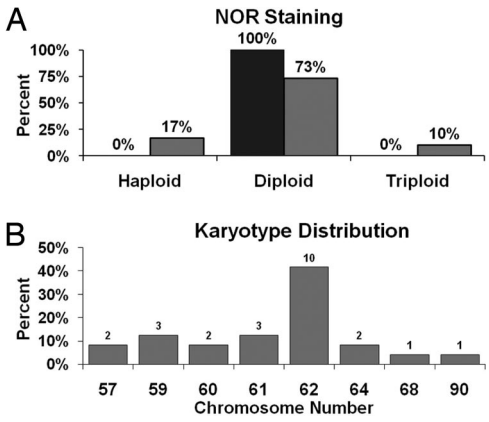

Fig. 4. Evaluation of rainbow trout embryonic cell nuclei by nucleolar organizer region (NOR) and karyotype analysis. (*A*) Percentage of individuals tested exhibiting 1, 2, or 3 NORs consistent with being monosomic, disomic, or trisomic for chromosome 20. (*B*) Karyotype distributions for 24 individuals propagated from male parents exposed to EE2. Individuals with fewer than or greater than 62 chromosomes represent a hypoploid or hyperploid condition, respectively, whereas those with 62 chromosomes represent a normal diploid condition. Chromosome numbers presented represent only those observed in this analysis.

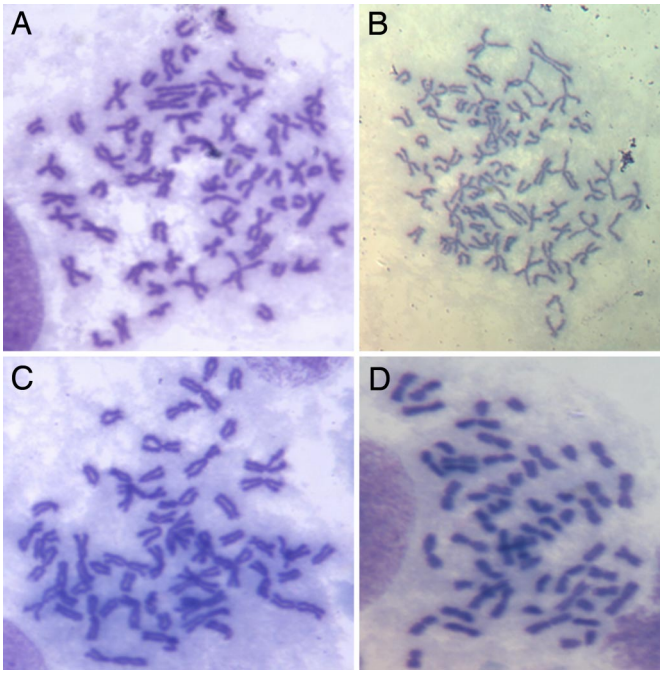

Fig. 5. Photomicrographs showing typical rainbow trout chromosome spreads used for karyotype analysis. (*A*) Normal, diploid cell; (*B*) hyperploid cell; and (*C* and *D*) hypoploid cells.

recombination sites, or chiasma, exhibit higher levels of aneuploidy in gametes (56–61).

Male rainbow trout exposed to EE2 exhibit high levels of aneuploid sperm but are capable of fertilizing eggs *in vitro*, which leads to the production of high levels of aneuploid embryos. Analysis of NOR staining indicated that chromosome 20 was involved in half of the aneuploidies. Although NOR staining cannot specifically determine hypoploidy/hyperploidy vs. monosomy/trisomy, the natural production of monosomic/trisomic individuals in rainbow trout is an exceedingly rare event when females are properly maintained (62). This fact combined with NOR staining results may indicate that chromosome 20 is somehow specifically susceptible to aneuploidy and/or EE2 exposure during meiosis. As mentioned previously, similar observations have been recorded in human sperm analyses where specific chromosomes are known to have a higher frequency of aneuploidy in normal, fertile males due to decreased meiotic recombination (56–59). This fact may be exacerbated in male rainbow trout because they exhibit the highest degree of recombination suppression in linkage map distance ratios among vertebrates (63), although it remains to be determined if and how estrogens interact and negatively impact recombination sites.

While human autosomal monosomy is considered extremely lethal based on an inability to detect it in clinically recognized pregnancies (64), we observed over two and one-half times the number of hypoploid embryos compared with hyperploid embryos. Several facts may account for this observation in rainbow trout. The first is that salmonid fishes, including the rainbow trout, underwent an autotetraploid event between 25–100 million years ago that duplicated their entire genome (65). Since this event salmonids have undergone Robertsonian rearrangements reducing their chromosome complement (66). Despite these rearrangements, genome duplication can be observed with additional alleles (beyond the normal two) having been identified for many genes (67–69). The presence of such additional alleles in rainbow trout may reduce detrimental effects of embryonic monosomy through some type of compensation, or partial compensation, which allows embryos to survive longer as a more equal level of transcript formation is maintained (i.e., 4:3 in trout vs. 2:1 in mammal monosomy). The physiological effects of hyperploidy may also be more detrimental than hypoploidy in rainbow trout. Torres and colleagues (70) recently reported in yeast that hyperploidy produced a general phenotype with decreased cell cycle progression and increased environmental sensitivity to protein synthesis and folding, among others. This general phenotype was observed with nearly all of the possible yeast hyperploid genotypes. Given the reduced number of hyperploid individuals in our study, an inhibition of growth due to hyperploidy in rainbow trout must be considered.

High levels of aneuploid sperm in EE2 exposed male rainbow trout was strongly correlated with embryonic aneuploidy in this study and was likely the cause of previously observed reductions in embryonic survival (31, 42). This discovery of increased aneuploid sperm formation has broad implications for all sexually mature, or actively spermatogenic, male vertebrates experiencing, or potentially experiencing, environmental estrogen exposure. Despite this advancement in understanding how environmental estrogens affect fertility/development, it remains to be determined what the molecular mechanism is at the level of the developing male germ cell. Because the developing male fish germ cell is sensitive to environmental estrogens, and the fishes are basal in the vertebrate lineage, it is suggestive of a mechanism widely conserved across vertebrate groups.

Materials and Methods

Trout Strain, EE2 Exposure, and Semen Collection. Fish used in these experiments were maintained according to guidelines established by the Institutional Animal Care and Use Committees of Battelle Pacific Northwest National Laboratory, Washington State University and the University of Idaho. Clonal, male Arlee strain rainbow trout (100% homozygous) were used. Fish were propagated at the Washington State University research fish hatchery using androgenesis (i.e., all paternal inheritance) (71). This process results in YY male fish with normal fertility despite lower levels of recombination observed through chromosome markers studies (63, 72). The experimental advantages of clonal rainbow trout are that they are genetically identical and all-male. At 16 months of age (late stage juvenile), 10 individuals were selected from a group previously transported to Marine Research Laboratory (Sequim, WA) and communally held in 370-liter fiberglass tanks with a single-pass flow-through freshwater system with water chemistry/quality parameters previously described (31). Fish were assigned randomly to one of two treatments, five for the control group and five for the chemical exposure group, and placed into 370-liter tanks. Chemical exposures were performed as described in Brown *et al.* (31). Water and stock solution in-flow rates for both treatments were monitored daily and measured by GCMS every 7–14 days. Exposures continued for 50 days after which semen samples were collected from all sexually mature individuals. Fish were anesthetized before sample collection using buffered 0.25 g/l MS-222 (Argent). Individual semen samples were collected by manual expression directly into sterile plastic bags (Whirl-Pak, NASCO) and placed on ice for transport to the University of Idaho. Upon arrival semen was cryopreserved by using standard salmonid sperm cryopreservation techniques with 10 0.5 ml of cryostraws per individual used (73, 74).

Sperm Fluorescent *in Situ* Hybridization (FISH). Three 0.5-ml cryopreserved semen samples from each fish sampled were removed from liquid nitrogen storage and thawed in warm tap water (20°C). The contents of all three cryostraws for each individual were combined in a 15-ml conical tube and fixed with 10 ml of a 3:1 methanol: glacial acetic acid solution. Samples were then centrifuged for 10 min at 2,000 rpm after which time the supernatant was removed and the process repeated two more times. After the final centrifuge cycle, 10 ml of 3:1 methanol: Glacial acetic acid solution was added and samples placed at −20°C or used to prepare FISH slides. Fixed sperm were dropped onto ethanol washed, dd H_2O wetted glass, microscope slides held at a 45° angle and dried over-night before being used in FISH or stored at −20°C. PCR amplification of DNA probes for 18s rDNA (75) and 5s rDNA, which is located on both sex chromosomes in rainbow trout (76) were carried out as reported. The amplified products were labeled with the fluorochrome-labeled dUTPs spectrum green (Vysis, Abbott Laboratories) and spectrum orange (Vysis) for the 18s and 5s rDNA, respectively, using a nick translation kit (Vysis) as described by the manufacturer. Denaturation, probe hybridization

and postwash protocols were carried out as described by the manufacturer (Vysis) with one minor modification. Specifically, no blocking agents (rainbow trout CoT DNA or nonspecies specific DNA) were added with labeled DNA to probe mixtures used for hybridization. After washing, slides were counterstained with DAPI/VECTASHIELD medium (Vector Laboratories) and a glass coverslip applied. Slides were examined using a Leica DMR compound microscope containing a laser light source, appropriate fluorescent filters and attached SPOT camera. A minimum of 500 sperm per individual was analyzed to determine the percent aneuploid sperm per sample.

Fertilization and Embryonic Tissue Preparations. Fertilizations were performed by using standard salmonid *in vitro* fertilization techniques using unfertilized eggs obtained from a single outbred rainbow trout female provided by TroutLodge Inc. and cryopreserved semen. *In vitro* fertilizations were performed by using semen samples from males of both treatment groups (control and EE2 exposed) as described by Cloud *et al.* (77). Nine days after fertilization, developing embryos were prepared for nucleolar organizer region (NOR) and chromosome staining as described in Thorgaard and Disney (78). Embryos were dissected from eggs in 0.9% saline and separated from the yolk before being cultured for 4 h at 19°C in PBS medium containing 25 μg/ml colchicine. After culture, embryos were transferred to a hypotonic solution (1% sodium citrate) for 30 min after which time the hypotonic solution was removed and embryos fixed twice in a freshly prepared 3:1 methanol:glacial acetic acid solution. Embryos were then stored in fixative for a minimum of 12 h at 4°C to ensure complete penetration of fixative. After complete fixation, slides were prepared by using the method described by Kligerman and Bloom (79) with prewarmed slides at 50°C for NOR staining or 45°C for karyotype analysis.

NOR Staining. A single NOR is present in the rainbow trout genome that localizes to chromosome 20 (48, 49). Staining to reveal NORs within the cell nuclei of individuals was performed by using the method proposed by Goodpasture and Bloom (80) and Howell and Black (81) as modified by Gold (82). This method has been previously used to analyze triploidy in rainbow trout and other salmonid fishes (49). For each individual 50 cells per slide, on two slides (total = 100 cells), were analyzed to determine marker ploidy.

Karyotype Analysis. Slides prepared for chromosome count staining, to determine karyotype, used the protocol described by Thorgaard and Disney (78). After staining slides were evaluated by using Nomarski optics on a Leica DMR compound microscope to identify chromosome spreads suitable for counting. When quality chromosome spreads were identified pictures were taken by using an attached SPOT camera with associated software to obtain digital images for establishment of the karyotype. The karyotype for each embryo analyzed was determined based on the chromosome count from 15 spreads with identical counts from at least 10 required to assign chromosome number. The karyotype for the rainbow trout cross in our study was 62, which is within the range of 58–64 and is consistent with all populations of rainbow trout having 104 chromosome arms (50).

Statistical Analysis. The percent of aneuploid sperm per 500 sperm cells counted was analyzed statistically by using a completely randomized design. The percentage of aneuploid sperm present in each sample was analyzed after performing an arcsine transformation to normalize values. The linear model for this analyses was $y_{ij} = \mu + \alpha_i + e_{ij}$, where α_i represents EE2 treatment effects and e_{ij} the random error. ANOVA was performed to determine whether significant differences were present between treatments using PROC GLM in SAS/STAT (SAS Institute).

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1. Kolpin DW, *et al.* (2002) Pharmaceuticals, hormones, and other organic wastewater contaminants in US streams, 1999–2000: A national reconnaissance. *Environ Sci Technol* 36:1202–1211.
2. Matthiessen P, *et al.* (2006) Contamination of headwater streams in the United Kingdom by oestrogenic hormones from livestock farms. *Sci Total Environ* 367:616–630.
3. Vandenberg LN, Hauser R, Marcus M, Olea N, Welshons WV (2007) Human exposure to bisphenol A (BPA). *Reprod Toxicol* 24:139–177.
4. Ackerman LK, *et al.* (2008) Atmospherically deposited PBDEs, pesticides, PCBs, and PAHs in Western US National Park fish: Concentrations and consumption guidelines. *Environ Sci Technol* 42:2334–2341.
5. Carlsen E. Giwercman A. Keiding N. Skakkebick NE (1992) Evidence tor decreasing quality of semen during past 50 years. *British Med J* 305:609–613.
6. Auger J, Kunstmann JM, Czyglik F, Jouannet P (1995) Decline in semen quality among fertile men in Paris during the past 20 years. *N Engl J Med* 332:281–285.
7. Irvine S, Cawood E, Richardson D, MacDonald E, Aitken J (1996) Evidence of deteriorating semen quality in the United Kingdom: Birth cohort study in 577 men in Scotland over 11 years. *British Med J* 312:467–471.
8. Swan SH, Elkin EP, Fenster L (1997) Have sperm densities declined? A reanalysis of global trend data. *Environ Health Perspect* 105:1228–1232.
9. Swan SH, Elkin EP, Fenster L (2000) The question of declining sperm density revisited: An analysis of 101 studies published 1934–1996. *Environ Health Perspect* 108:961–966.
10. Giwercman A, Bonde JP (1998) Declining male fertility and environmental factors. *Endocrinol Metab Clin North Am* 27:807–830.
11. Petrelli G, Mantovani A (2002) Environmental risk factors and male fertility and reproduction. *Contraception* 65:297–300.
12. Olsen GW, Bodner KM, Ramlow JM, Ross CE, Lipshultz LI (1995) Have sperm counts been reduced 50-percent in 50 years-A statistical-model revisited. *Fertil Steril* 63:887–893.
13. Fisch H, *et al.* (1996) Semen analyses in 1,283 men from the United States over a 25-year period: No decline in quality. *Fertil Steril* 65:1009–1014.
14. Vierula M, *et al.* (1996) High and unchanged sperm counts of Finnish men. *Intl J Androl* 19:11–17.
15. Czeizel A, Toth J, Czvenits H (1986) Increased birth prevalence of isolated hypospadias in Hungary. *Acta Paediatr Hung* 27:329–337.
16. Sharpe RM, Skakkebaek NE (2003) Male reproductive disorders and the role of endocrine disruption: Advances in understanding and identification of areas for future research. *Pure Appl Chem* 75:2023–2038.
17. Boisen KA, *et al.* (2004) Difference in prevalence of congenital cryptorchidism in infants between two Nordic countries. *Lancet* 363:1264–1269.
18. Richiardi L, *et al.* (2004) Testicular cancer incidence in eight Northern European countries: Secular and recent trends. *Cancer Epidemiol Biomarkers Prev* 13:2157–2166.
19. Anway MD, Cupp AS, Uzumcu M, Skinner MK (2005) Epigenetic transgenerational actions of endocrine disruptors and male fertility. *Science* 308:1466–1469.
20. Dolinoy DC, Huang D, Jirtle RL (2007) Maternal nutrient supplementation counteracts bisphenol A-induced DNA hypomethylation in early development. *Proc Natl Acad Sci USA* 104:13056–13061.
21. Klotz DM, *et al.* (1996) Identification of environmental chemicals with estrogenic activity using a combination of in vitro assays. *Environ Health Perspect* 10:1084–1089.
22. Tyler C, Jobling S, Sumpter JP (1998) Endocrine disruption in wildlife: A critical review of the evidence. *Crit Rev Toxicol* 28:319–363.
23. Sonnenschein C, Soto AM (1998) An updated review of environmental estrogen and androgen mimics and antagonists. *J Steroid Biochem Mol Biol* 65:143–150.
24. Mills LJ, Chichester C (2005) Review of evidence: Are endocrine-disrupting chemicals in the aquatic environment impacting fish populations? *Sci Total Environ* 343:1–34.
25. Jobling S, Nolan M, Tyler CR, Brighty G, Sumpter JP (1998) Widespread sexual disruption in wild fish. *Environ Sci Technol* 32:2498–2506.
26. Jobling S, *et al.* (2002) Wild intersex roach (*Rutilus rutilus*) have reduced fertility. *Biol Reprod* 67:515–524.
27. Aravindakshan J, Gregory M, Marcogliese DJ, Fournier M, Cyr DG (2004) Consumption of xenoestrogen-contaminated fish during lactation alters adult male reproductive function. *Toxicol Sci* 81:179–189.
28. Rhind SM (2005) Are endocrine disrupting compounds a threat to farm animal health, welfare and productivity? *Reprod Domest Anim* 40:282–290.
29. Woodling JD, Lopez EM, Maldonado TA, Norris DO, Vajda AM (2006) Intersex and other reproductive disruption of fish in wastewater effluent dominated Colorado streams. *Comp Biochem Physiol C Toxicol Pharmacol* 144:10–15.
30. Assinder S, Davis R, Fenwick M, Glover A (2007) Adult-only exposure of male rats to a diet of high phytoestrogen content increases apoptosis of meiotic and post-meiotic germ cells. *Reproduction* 133:11–19.
31. Brown KH, Schultz IR, Nagler JJ (2007) Reduced embryonic survival in rainbow trout resulting from paternal exposure to the environmental estrogen 17α-ethynylestradiol during late sexual maturation. *Reproduction* 134:659–666.
32. Maunder RJ, Matthiessen P, Sumpter JP, Pottinger TG (2007) Impaired Reproduction in Three-Spined Sticklebacks Exposed to Ethinyl Estradiol as Juveniles. *Biol Reprod* 77:999–1006.
33. McClusky LM, de Jager C, Bornman MS (2007) Stage-related increase in the proportion of apoptotic germ cells and altered frequencies of stages in the spermatogenic cycle following gestational, lactational, and direct exposure of male rats to p-nonylphenol. *Toxicol Sci* 95:249–256.
34. Cheshenko K, Pakdel F, Segner H, Kah O, Eggen RIL (2008) Interference of endocrine disrupting chemicals with aromatase CYP19 expression or activity, and consequences for reproduction of teleost fish. *Gen Comp Endocrinol* 155:31–62.
35. Kaneto M, Kanamori S, Hishikawa A, Kishi K (1999) Epididymal sperm motion as a parameter of male reproductive toxicity: Sperm motion, fertility, and histopathology in ethinylestradiol-treated rats. *Reprod Toxicol* 13:279–289.
36. Rozati R, Reddy PP, Reddanna P, Mujtaba R (2002) Role of environmental estrogens in the deterioration of male factor fertility. *Fertil Steril* 78:1187–1194.
37. Takahashi O, Oishi S (2003) Testicular toxicity of dietarily or parenterally administered bisphenol A in rats and mice. *Food Chem Toxicol* 41:1035–1044.
38. Maffini MV, Rubin BS, Sonnenschein C, Soto AM (2006) Endocrine disruptors and reproductive health: The case of bisphenol-A. *Mol Cell Endocrinol* 254:179–186.

39. Fry DM (1995) Reproductive effects in birds exposed to pesticides and industrial-chemicals. *Environ Health Perspect* 103:165–171.
40. Pacchierotti F, Ranaldi R, Eichenlaub-Ritter U, Attia S, Adler ID (2008) Evaluation of aneugenic effects of bisphenol A in somatic and germ cells of the mouse. *Mutat Res* 651:64–70.
41. Bennetts LE, *et al.* (2008) Impact of estrogenic compounds on DNA integrity in human spermatozoa: Evidence for cross-linking and redox cycling activities. *Mutat Res* 641:1–11.
42. Schultz IR, Skillman A, Nicolas J, Cyr DG, Nagler JJ (2003) Short-term exposure to 17a-ethynylestradiol decreases the fertility of sexually maturing male rainbow trout (Oncorhynchus mykiss). *Environ Toxicol Chem* 22:1272–1280.
43. Thorgaard GH, Scheerer PD, Parsons JE (1985) Residual paternal inheritance in gynogenetic rainbow trout: Implications for gene transfer. *Theor Appl Genet* 71:119–121.
44. Disney JE, Johnson KR, Thorgaard GH (1987) Intergeneric gene transfer of six isozyme loci in rainbow trout by sperm chromosome fragmentation and gynogenesis. *J Exp Zool* 244:151–158.
45. Patton SJ, Kane SL, Wheeler PA, Thorgaard GH (2007) Maternal and paternal influence on early embryonic survival of androgenetic rainbow trout (*Oncorhynchus mykiss*): Implications for measuring egg quality. *Aquaculture* 263:26–34.
46. Hunt PA, *et al.* (2003) Bisphenol A exposure causes meiotic aneuploidy in the female mouse. *Curr Biol* 13:546–553.
47. Eichenlaub-Ritter U, *et al.* (2007) 2-Methoxyestradiol induces spindle aberrations, chromosome congression failure, and nondisjunction in mouse oocytes. *Biol Reprod* 76:784–793.
48. Phillips RB, *et al.* (2006) Assignment of rainbow trout linkage groups to specific chromosomes. *Genetics* 174:1661–1670.
49. Phillips RB, Zajicek KD, Ihssen PE, Johnson O (1986) Application of silver staining to the identification of triploid fish cells. *Aquaculture* 54:313–319.
50. Thorgaard GH (1983) Chromosomal differences among rainbow-trout populations. *Copeia* 1983:650–662.
51. Carrell DT, *et al.* (2004) Sperm chromosome aneuploidy as related to male factor infertility and some ultrastructure defects. *Arch Androl* 50:181–185.
52. Collodel G, Moretti E (2008) Morphology and meiotic segregation in spermatozoa from men of proven fertility. *J Androl* 29:106–114.
53. Monteil M, *et al.* (1997) Increased aneuploid frequency in spermatozoa from a Hodgkin's disease patient after chemotherapy and radiotherapy. *Cytogenet Cell Genet* 76:134–138.
54. Attia SM, Badary OA, Hamada FM, Hrabe de Angelis M, Adler I (2008) The chemotherapeutic agents nocodazole and amsacrine cause meiotic delay and non-disjunction in spermatocytes of mice. *Mutat Res* 651:105–113.
55. Wiland E, Midro AT, Panasiuk B, Kurpisz M (2007) The analysis of meiotic segregation patterns and aneuploidy in the spermatozoa of father and son with translocation t(4;5)(p15.1;p12) and the prediction of the individual probability rate for unbalanced progeny at birth. *J Androl* 28:262–272.
56. Kirkpatrick G, *et al.* (2008) A comparison of sperm aneuploidy rates between infertile men with normal and abnormal karyotypes. *Hum Reprod* 23:1679–1683.
57. Furguson KA, Wong EC, Chow V, Nigro M, Ma S (2007) Abnormal meiotic recombination in infertile men and its association with sperm aneuploidy. *Hum Mol Genet* 16:2870–2879.
58. Sun F, *et al.* (2007) Abnormal progression through meiosis in nonobstructive azoospermia. *Fertil Steril* 87:565–571.
59. Sun F, *et al.* (2008) The relationship between meiotic recombination in human spermatocytes and aneuploidy in sperm. *Hum Reprod* 23:1691–1697.
60. Hassold TJ, Hunt PA (2001) To err (meiotically) is human: The genesis of human aneuploidy. *Nat Rev Genet* 2:280–291.
61. Martin RH (2005) Mechanisms of nondisjunction in human spermatogenesis. *Cytogenet Genome Res* 111:245–249.
62. Aegerter S, Jalabert B (2004) Effects of post-ovulatory oocyte ageing and temperature on egg quality and on the occurrence of triploid fry in rainbow trout, *Oncorhynchus mykiss*. *Aquaculture* 231:59–71.
63. Sakamoto T, *et al.* (2000) A microsatellite linkage map of rainbow trout (*Oncorhynchus mykiss*) characterized by large sex-specific differences in recombination rates. *Genetics* 155:1331–1345.
64. Hartl DL, Jones EW (2002) in *Essential genetics: A genomics perspective*, eds Hartl DL Jones EW (Jones and Bartlett Publishers, Boston), pp 177–178.
65. Allendorf FW, Thorgaard GH (1984) in *The Evolutionary Genetics of Fishes*, ed Turner BJ (Plenum, New York), pp 1–53.
66. Thorgaard GH (1976) Robertsonian polymorphism and constitutive heterochromatin distribution in chromosomes of the rainbow trout (*Salmo gairdneri*). *Cytogenet Cell Genet* 17:174–184.
67. Brunelli JP, Robison BD, Thorgaard GH (2001) Ancient and recent duplications of the rainbow trout Wilms' tumor gene. *Genome* 44:455–462.
68. Moghadam HK, Ferguson MM, Danzmann RG (2005) Evidence for Hox gene duplication in rainbow trout (*Oncorhynchus mykiss*): A tetraploid model species. *J Mol Evol* 61:804–818.
69. Nagler JJ, Cavileer T, Sullivan J, Cyr DG, Rexroad C (2007) The complete nuclear estrogen receptor family in the rainbow trout: Discovery of the novel ER alpha 2 and both ER beta isoforms. *Gene* 392:164–173.
70. Torres EM, *et al.* (2007) Effects of aneuploidy on cellular physiology and cell division in haploid yeast. *Science* 317:916–924.
71. Parsons JE, Thorgaard GH (1985) Production of androgenetic diploid rainbow trout. *J Hered* 76:177–181.
72. Nichols KM, *et al.* (2003) A consolidated linkage map for rainbow trout (*Oncorhynchus mykiss*). *Anim Genet* 34:102–115.
73. Stoss J, Holtz W (1983) Cryopreservation of rainbow trout (*Salmo gairdneri*) sperm. IV. The effect of DMSO concentration and equilibration time on sperm survival; sucrose and KCl as extender components and the osmolality of the thawing solution. *Aquaculture* 32:321–330.
74. Scheerer PD, Thorgaard GH (1989) Improved fertilization by cryopreserved rainbow trout semen with theophylline. *The Progressive Fish-Culturist* 51:179–182.
75. Domanico MJ, Phillips RB, Oakley TH (1997) Phylogenetic analysis of Pacific salmon (genus *Oncorhynchus*) using nuclear and mitochondrial DNA sequences. *Can J Fish Aquat Sci* 54:1865–1872.
76. Phillips RB, Noakes MA, Morasch M, Felip A, Thorgaard GH (2004) Does differential selection on the 5S rDNA explain why the rainbow trout sex chromosome heteromorphism is not linked to the SEX locus? *Cytogenet Genome Res* 105:122–125.
77. Cloud JG, Miller WH, Levanduski MJ (1990) Cryopreservation of sperm as a means to store salmonid germplasm and to transfer genes from wild fish to hatchery populations. *The Progressive Fish-Culturist* 52:51–53.
78. Thorgaard GH, Disney JE (1990) in *Methods for Fish Biology*, eds Schreck CB, Moyle PB (American Fisheries Society, Bethesda) pp 171–190.
79. Kligerman AD, Bloom SE (1977) Rapid chromosome preparations from solid tissues of fishes. *J Fish Res Board Can* 34:266–269.
80. Goodpasture C, Bloom SE (1975) Visualization of nucleolar organizer regions in mammalian chromosomes using silver staining. *Chromosoma* 53:37–50.
81. Howell WM, Black DA (1980) Controlled silver staining of nucleolus organizer with a protective colloidal developer: A 1-step method. *Experientia* 36:1014–1045.
82. Gold JR (1984) Silver staining and heteromorphism of chromosomal nucleolus organizer regions in North American cyprinid fishes. *Copeia* 1984:133–139.